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Examiner	:	F.C. Prats
Art Unit	:	1651
Applicant	:	Bruce Joseph Roser
Serial No.	:	08/875,796
Filed	:	October 30, 1998
For	:	Dried Blood Factor Compositions Comprising Trehalose

DECLARATION OF ALAN P. MACKENZIE UNDER 37 CFR §1.132

I, hereby declare that:

I have the following educational background:

Univ. of London, England	B.Sc.	1951	General Science
Univ. of London, England	B.Sc.	1952	Chemistry
Univ. of London, England	Ph.D.	1957	Chemistry

Honors: Both B.Sc. degrees were taken with First Class Honours.

I have held the following Research and Professional Appointments:

Research Associate Professor, Center for Bioengineering, School of Medicine, University of Washington, Seattle, Washington.

Associate Professor, Center for Bioengineering, School of Medicine, University of Washington, Seattle, Washington.

Research Associate, High Voltage Electron Microscope Laboratory, University of Wisconsin, Madison, Wisconsin.

Associate Director, Cryobiology Research Institute, Madison, Wisconsin.

Associate Director of Research, American Foundation for Biological Research, Madison, Wisconsin.

Research Associate, American Foundation for Biological Research, Madison, Wisconsin.

Staff Scientist, Glaxo Research, Glaxo Laboratories, Ltd., Greenford, Middlesex, England.

The following are representative of my technical publications:

MacKenzie, A.P. and Luyet, B.J.: A collodion sandwich-film technique for the study of the growth of ice in very thin layers of aqueous solutions. In "Electron Microscopy, Vol. 2" (Proceedings of the Fifth International Congress for Electron Microscopy, Philadelphia, 1962). Ed.: S.S. Breese, Academic Press, New York, N.Y., p. P 2, 1962.

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D. Nochlin, A. P. MacKenzie, E. M. Bryant, I. H. Norwood, and S. M. Sumi. 1993, A Simple Method of Rapid Freezing Adequately Preserves Brain Tissue for Immunocytochemistry, Light and Electron Microscopic Examination. Acta Neuropathologica, 86, 645-650.

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W.R. Gombotz, S. C. Pankey, L. S. Bouchard, D. H. Phan, and A. P. MacKenzie. 1996.Stability, Characterization, Formulation, and Delivery System Development for transforming Growth Factor-Beta. In: Formulation, Characterization, and Stability of Protein Drugs, R. Pearlman and Y. J. Wang, Editors, Plenum Press, New York, pp. 219-245.

The following is a brief, but accurate, synopsis of my relevant experience:

Dr. MacKenzie began his continuous career in freezing and freeze-drying in 1959. His earliest studies centered on the fundamental aspects of the freezing and freeze-drying processes. Later his work focused also on pharmaceutical, microbiological and diagnostic applications. Dr. MacKenzie has published more than 50 papers on lyophilization and presented more than 100 papers from the platform at national and international meetings. Dr. MacKenzie has developed and currently maintains a practical and theoretical interest in the biophysics and hydration of peptides and proteins and their freezing and freeze-drying behavior.

I have read and understood the specification and claims of the subject application, the Office Action dated July 19, 2000, and the Office Action dated September 20, 2001;

and, being as duly qualified, do further declare:

I. The present invention is based on the discovery that Factor VIII compositions can be prepared in a stable dried form by freeze-drying an aqueous solution of Factor VIII using trehalose as a stabilising agent in the absence of albumin.

II. I have been asked to review an issued United States patent to determine its relevance to the present invention. Specifically, I have reviewed the following reference:

Livesey *et al.* (U.S. Patent No. 5,364,756)

III. I have carefully reviewed this reference and, for the reasons discussed below, conclude that there is no teaching or suggestion in this reference of the present invention.

IV. My review of this reference has been done in the context of what was known in the art when the present invention was made. My knowledge in this regard is based on many years of experience in this field dating back to 1959. In 1991, various Factor VIII compositions were known. Factor VIII preparations which were derived from human blood necessarily contained albumin. Although there were significant health risks associated with administering albumin to a patient, namely the potential risk for viral contaminants to be present, the presence of albumin was believed to be necessary in order to stabilise the Factor VIII protein. Recombinant Factor VIII preparations were also being prepared, but again it was believed to be necessary to add albumin to the preparations to stabilise the proteins. The presence of albumin was believed to be necessary because Factor VIII proteins are extremely labile, even in the presence of other stabilisers.

V. The teachings of the cited reference are entirely consistent with what was generally believed in the art at that time — that albumin was necessary to stabilise Factor VIII compositions. Although the presence or absence of albumin in a Factor VIII composition was clearly not the main focus of this reference, a careful review of this reference reveals evidence which supports the proposition that one skilled in the art would not have expected to be able to freeze dry Factor VIII in the absence of albumin.

VI. The Livesey *et al.* patent describes the cryopreservation of biological materials. The method and apparatus described by Livesey *et al.* can purportedly be used for cryopreservation of materials ranging from viruses to cultured mammalian cells. However, it is apparent from the disclosure, including the examples, that the primary focus is on the preservation of whole cells. Red blood cells, plateletes, leukocytes, sperm, pancreatic islets, and marrow cells are all listed as specific examples of cells which can be preserved using the Livesey *et al.* procedures. There is very little discussion, and there are no examples, of the preservation of proteins. Those skilled in the art know that materials and procedures used to preserve whole cells and/or viruses are not necessarily applicable to the stabilisation of proteins. Furthermore, Livesey *et al.* state that the exact ingredients of the suspensions which are to be preserved "is not considered to be a component of the invention." Thus, I find no disclosure in the Livesey *et al.* patent which is specifically relevant to the selection of appropriate stabilising agents for delicate proteins.

VII. Freeze drying of biological materials is a very complex, and poorly understood, process. Livesey *et al's.* extensive discussion of this process contains much information which is not relevant to the current review. However, the critical differences between ambient drying and cold temperature drying are expressly acknowledged by Liversey *et al.* It is clear from the discussion in the Livesey *et al.* reference that protection of proteins from damage due to freezing involves very different mechanisms from protecting proteins from damage due to drying. Livesey *et al.* contrast the role of cryoprotectants with dry protectants stating that "[t]he cryosolution may also include exposing the biological suspension to one or more dry protectant compounds. Dry protectants, by definition, stabilize samples in the dry state." Livesey *et al.* specifically list human serum albumin as a cryoprotectant which has been found to be effective in combination with trehalose (a dry protectant). Thus, the Livesey *et al.* reference does not teach that trehalose can be used in the absence of albumin.

VIII. Trehalose is only mentioned by Livesey *et al.* as one possible ingredient in a drying process. There is no reference to preparing a Factor VIII composition in the absence of albumin, and it cannot be inferred, given the knowledge at that time, that albumin was not required. Thus, the Livesey *et al.* discussion of stabilising agents is entirely consistent with the proposition that Factor VIII cannot be freeze-dried without the use of albumin as a stabilising agent. In my opinion, there is nothing in the Livesey *et al.* specification or claims which teaches, or even suggests, to those skilled in the art, that Factor VIII can be freeze-dried in the absence of albumin.

The undersigned declares further that all statements made herein of his own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements, and the like so made, are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the Application or any Patent issuing thereon.

Further declarant sayeth naught

Signed:

Date: 10/18/01

Products currently available for use in hemophilia A

Cryoprecipitate. Prepared from single plasma units by cold precipitation and resuspension in plasma. Although the source plasma is screened for HIV 1 and 2, anti-HBc, ALT, and anti-HCV, there is no viral inactivation step for the cryoprecipitate normally obtained from your local blood bank. Because of the theoretical risk of HIV infection, the use of cryoprecipitate to treat hemophilia is not recommended. The New York Blood Center is currently working on techniques to treat cryoprecipitate using solvent-detergent techniques but this is not currently available.

Intermediate purity factor VIII. Prepared from pooled human plasma which is screened for anti-HIV 1 and 2, ALT, anti-HBc, anti-HTLV I/II, and anti-HCV. Viral inactivation steps are used in all intermediate purity concentrates although the techniques differ from product to product. These inactivation steps generally appear to be highly effective against HIV and selected hepatitis viruses. The U. S. Hemophilia/HIV Seroconversion Surveillance Project sponsored jointly by the Centers for Disease Control, the Food and Drug Administration, and the National Hemophilia Foundation has shown no HIV seroconversions in hemophilia attributable to factor concentrates since 1987 and no hepatitis B or C seroconversions in hemophilia attributable to factor concentrates since 1992.

Intermediate purity factor VIII products which are currently available:

- **Factor VIII SD** (NY Blood Center; Melville) - Viral inactivation by extraction with TNBP - sodium cholate.
- **Humate-P** (Behringwerke) - Pasturized product prepared by heat treatment in solution to 60°C for 10 hours.
- **Profilate OSD** (Alpha) - Viral inactivation using TNBP - polysorbate 80.
- **Koate HP** (Bayer-Miles) - Viral inactivation using TNBP - polysorbate 80.
- **MelATE** (Melville) - Viral inactivation using TNBP - polysorbate 80.
- **Alphanate** (Alpha) - Purified from plasma by affinity chromatography using heparin-agarose to bind von Willebrand factor which is complexed with factor VIII. Viral inactivation using TNBP - polysorbate 80.

Monoclonal antibody purified factor VIII. Prepared from pooled human plasma which is screened for anti-HIV 1 and 2, anti-HBc, ALT, anti-HTLV I/II, and anti-HCV. The factor VIII is purified by affinity chromatography using mouse monoclonal antibodies to human factor VIII. The purified factor VIII prior to formulation has a specific activity of ~3000 units per mg. Human albumin is used as a stabilizer in the formulation of the factor VIII.

Monoclonal factor VIII products which are currently available:

- **Monoclate-P** (Centeon) - Prepared from commercial source plasma.
- **Hemofil-M** (Baxter) - Prepared from commercial source plasma. Affinity purified using a mouse monoclonal antibody which recognizes the factor VIII heavy chain.
- **AHF-ARC** (Red Cross) - Prepared from Red Cross volunteer source plasma and purified and formulated at Baxter using the same methods used to purify Hemofil-M.

Recombinant factor VIII. Recombinant factor VIII is a synthetic form of factor VIII prepared in mammalian cells, such as Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cells. These are immortalized cell lines which are stable in culture, and permit the high level expression of human proteins with expected post-translational modifications such as tyrosine sulfation and carbohydrate attachment. Initial concerns about the possible immunogenicity of recombinant factor VIII have been somewhat alleviated by 1) studies in previously-treated patients showing a very low prevalence of inhibitors, 2) studies in previously-untreated patients (PUPs) showing a higher but stable prevalence of inhibitors, and 3) studies in newborn and transgenic mice showing a lack of immunogenicity of recombinant factor VIII compared with plasma factor VIII. Because both products are formulated with human albumin, even though the albumin is pasturized, recombinant factor VIII is subject to the same recalls as plasma-derived factor VIII in the event that donors with Creutzfeld-Jacob disease contribute to the source plasma from which the albumin is prepared. Two forms of recombinant factor VIII are currently licensed for use.

Recombinant factor VIII products which are currently available:

- **Recombinate** (Baxter) - The full length cDNA for factor VIII is used to synthesize Recombinate. Synthesis is in Chinese hamster ovary (CHO) cells and the factor VIII is purified by monoclonal affinity affinity chromatography using a mouse anti-factor VIII antibody which recognizes the 90 kDa heavy chain of factor VIII. The purified factor VIII is formulated with pasturized human albumin as a stabilizer. No viral inactivation step is used in Recombinate. Licensed by the FDA in December 1992.

- **Kogenate** (Bayer-Miles) - The full length cDNA for factor VIII is synthesized in baby hamster kidney (BHK) cells, purified by monoclonal affinity chromatography using a mouse anti-factor VIII antibody that recognizes the x domain of factor VIII, and formulated with pasturized human albumin. No viral inactivation step is used in Kogenate. Licensed by the FDA in March 1993.

- **Bioclate** (Centeon) - identical with Baxter Recombinate and distributed by Centeon under a licensing agreement on the patent for factor VIII.

- **Helixate** (Centeon) - identical with Bayer-Miles Kogenate and distributed by Centeon under a licensing agreement on the patent for factor VIII.

The Medical and Scientific Advisory Committee (MASAC) recommends that individuals with hemophilia and their providers should consider the use of recombinant derived clotting factor as the first choice for replacement therapy.

Click here for more information on product safety and product recalls from the Food and Drug Administration.

Click here to return to the UNC Hemophilia Center Home Page.

Recombinant Protein Drugs
ed. by P. Buckel
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Clotting factors VIII and IX

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Introduction

Haemophilia is the most common congenital disorder of coagulation and affects approximately 1 in 10,000 males around the world. Haemophilia A is due to a deficiency of factor VIII in the circulating blood whilst haemophilia B (also known as Christmas disease) is a clinically identical disorder caused by factor IX deficiency. It is less common than haemophilia A and affects 1 in about 30,000 males. Both factors VIII and IX are essential glycoproteins in the clotting cascade [1] (Fig. 1). The hallmark of severe haemophilia is recurrent and spontaneous haemarthrosis, typically affecting the hinge joints such as the ankle, knee and elbow. The severity of bleeding depends upon the level of factor in the blood.

Severe haemophilia is usually defined by a level of <2 iu/dl (or <2%) of factor VIII or IX in plasma. Moderately severely affected patients have levels varying from 2–5 iu/dl and mild from 5–25 iu/dl. It is unusual for an infant to have spontaneous haemarthroses in the first few months of life, and the first joint to be affected tends to be the ankle as the child learns to crawl. Repeated bleeding into joints may cause permanent damage, with painful arthritis and limb deformity and associated muscle wasting. Bleeding into muscles is also a feature of haemophilia, but this is usually a consequence of direct injury, albeit often minor. Bleeds into certain areas are particularly dangerous because of the risk of compression of neighbouring structures. Bleeds in the tongue can obstruct the airway, and retroperitoneal bleeding within the ilio-psoas muscle may result in femoral nerve compression. Bleeding from the gastrointestinal tract and bleeding into the urinary tract may also occur. There is also a significant risk of intracranial haemorrhage in severe haemophilia which was a significant cause of mortality in the past when treatment was not so readily available. The poor prognosis in the absence of effective treatment is reflected by the fact that the median life expectancy of haemophiliacs in Sweden was only 11 years during the period 1831–1920, but rose to 56.8 years during the period 1961–1980 [2]. It is also interesting to note that a century ago haemophilia affected several members of the royal families of Britain, Spain and Russia,

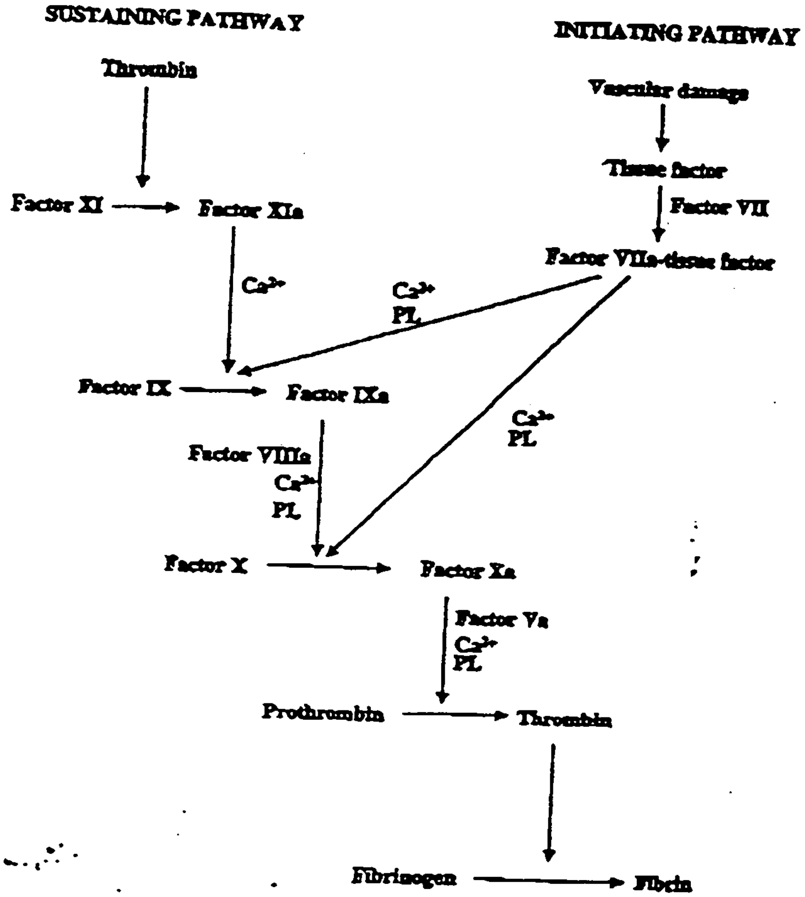

Figure 1. The revised clotting cascade. Thrombin, formed by the action of factor Xa as a result of vascular damage in the initiating (or extrinsic) pathway activates factor XI, thereby sustaining the cascade through the sustaining (or intrinsic) pathway. PL = phospholipid. The scheme is simplified and omits many details for clarity [1].

but, despite the best medical facilities of the age being available, all affected members died in their youth and no surviving descendants are affected.

Approximately 5% of patients with haemophilia A develop inhibitory antibodies to factor VIII at some stage but it is quite likely that this figure underestimates the true prevalence. Inhibitor development in haemophilia B is, by contrast, very rare (<1%). This is a potentially serious complication of therapy, as patients are refractory to conventional doses of coagulation factor concentrates and bleeding can be difficult to control. Family studies suggest that there is a genetic predisposition to the formation of antibodies but no HLA association or other linkages have been conclusively identified. There is some evidence that people of Afro-Caribbean origin are more susceptible to inhibitor formation than other ethnic groups. Certain mutations are undoubtedly associated with a significantly increased risk of inhibitor development, particularly large gene deletions and nonsense mutations resulting in stop codons – presumably because patients lack immune tolerance [3, 4]. There is a weaker association between inhibitor formation and the presence of an inversion in intron 22 of the factor VIII gene [5].

The genes for factor VIII and IX are both located at the telomere of the long arm of the X chromosome and thus haemophilia is inherited as an X-linked, recessive condition affecting males. The daughters of affected males are obligate carriers but sons are normal. A proportion of all cases of haemophilia occurs in the absence of a previous family history and is due to new mutations. Perhaps the most famous example is that of Queen Victoria, who had a haemophiliac son (Leopold) and also two daughters who were carriers. It is not known, however, whether they suffered from haemophilia A or B. There are very rare instances of haemophilia affecting females due to inheritance of the defective gene from both parents or to unequal X-inactivation in carriers [6]. There are also reports of haemophilia in females with Turner's syndrome (XO karyotype) and congenital androgen insensitivity (testicular feminization with XY karyotype).

Molecular basis of haemophilia A

Factor VIIIa is an essential cofactor which is required for the activation of factor X by factor IXa in the clotting cascade (Fig. 1). Factor VIII is a glycoprotein of 2332 amino acids and is synthesized predominantly by hepatocytes of the liver. It is processed intracellularly in the Golgi apparatus by proteolytic cleavage giving rise to a N-terminal heavy chain and a C-terminal light chain. Its domain structure (Fig. 2A) includes a carbohydrate-rich B domain that is not required for activity (see below). Factor VIII is activated to give rise to factor VIIIa by further proteolytic cleavage, probably by thrombin. In plasma, factor VIII circulates as a large glycoprotein complex non-covalently bound to multimers of von Willebrand factor (Fig. 2). The factor VIII gene is about 186 kb in length, with 26 exons, and is situated on the long arm of the X chromosome at Xq28.

Developments in molecular biology have permitted rapid identification of mutations in haemophilia A (and B) patients by various methods. PCR amplification of either genomic DNA or cDNA derived from the reverse transcription of mRNA of patient lymphocytes has superseded older methods based on restriction enzyme digestion and Southern blotting. Although automated DNA sequence analysers have been developed, gene sequencing of the entire factor VIII gene would be both expensive and labour-intensive because of its size.

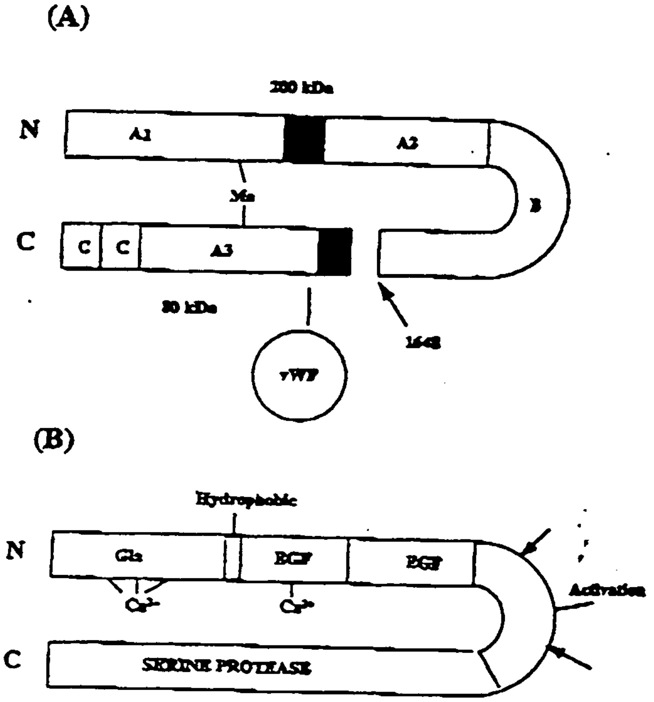

Figure 2. The domain structure of (A) factor VIII and (B) factor IX. In factor VIII (above) the N-terminal heavy chain and C-terminal light chain, formed after proteolysis at amino acid 1648 as shown, are stabilized by a metal (Me) binding site; a proposed site of binding to von Willebrand (vWF) factor is shown. The A1 and 2, B and C domains are marked. In factor IX (below) arrows mark the sites of proteolytic cleavage in the activation domain by either factor XIa or VIIa. For further details, see text.

Thus methods have been developed to initially prescreen the gene or mRNA, in order to define the mutated exon of the DNA, or region of the mRNA, before sequencing to define the actual mutation itself. Chemical cleavage of mismatches in heteroduplexes formed from the mutated DNA and a test DNA is the best current technique for detecting all mutations [7, 8]. Other useful methods are conformation-sensitive gel electrophoresis and denaturing-gradient gel electrophoresis, although these alternatives do not allow all mutations to be detected.

By far the commonest single genetic defect causing haemophilia A is an inversion in intron 22, which is encountered in about 20% of all patients, and in nearly half of all severely affected patients [5]. The inversion mechanism proposed involves homologous, intra-chromosomal recombination between an intronless gene of unknown function, designated F8A, which lies within intron

22 of the factor VIII gene and either of 2 further copies of F8A flanking the factor VIII gene. In either case, the inversion results in a truncated factor VIII gene generating a truncated mRNA and inactive factor VIII protein, thus explaining the severe haemophilia. Recombination with the distal copy of F8A is commoner than with the proximal copy and accounts for approximately 80% of the inversions (reviewed in [9]). It is now recognised that inversion is much more common in a male than a female meiosis.

All other known haemophilia A mutations are summarized in a valuable mutation database [10]. At present, 454 unique mutations are listed, including 92 cases of large deletions. However, it is generally thought that large deletions account for only a minority of cases of haemophilia and the reason that a much higher proportion than 5% is listed in the database reflects an ascertainment bias. Most mutations are point mutants or very short additions or deletions of only a few nucleotides. Nonsense, frameshift and splice-site mutations, caused by point mutations or short additions or deletions, usually cause severe haemophilia. Most other cases are caused by missense mutations causing amino acid substitutions and 218 missense mutations are presently known [10]. They have varying degrees of severity for patients from very mild to very severe [10]. Mutations at CG doublets, giving rise to a TG or CA doublet, are common. As is well known, CG doublets are genomic "hotspots" because of specific methylation of cytosine to 5' methylcytosine and because the methylated cytosine is particularly susceptible to mutation by deamination.

Molecular basis of haemophilia B

Factor IXa is a serine protease required for the activation of factor X in the clotting cascade. Factor IXa is itself formed from factor IX by proteolysis by either factor VIIa-tissue factor complex, which initiates the clotting cascade, or by factor XIa, which sustains it [1] (Fig. 1). Factor IX is a glycoprotein of 415 amino acids and is made up of a N-terminal γ-carboxyglutamic acid-rich sequence (Gla domain), two epidermal growth factor-like (EGF) domains – the first of which binds calcium – an activation domain and finally a C-terminal serine protease or catalytic domain (Fig. 2B). The 12 glutamic acid residues of the Gla domain undergo γ-carboxylation by a vitamin K-dependent γ-glutamyl carboxylase in the endoplasmic reticulum (ER) during the synthesis of factor IX in the liver hepatocyte. This essential post-translational modification is necessary for the correct protein folding and calcium binding of factor IX. The factor IX gene is about 34 kb in length and contains eight exons. Its basic exon structure is similar in organisation to coagulation factors VII, X and protein C and it is likely that they all evolved from a common ancestral gene by gene duplication.

The factor IX gene is considerably smaller than that of factor VIII, and patients with haemophilia B have been studied more extensively than those with haemophilia A. The first defects identified in haemophilia B were large gene deletions detected in patients with inhibitory antibodies by Southern blotting [3]. However, it is now clear that point mutations, or short additions or deletions, account for the vast majority of cases of haemophilia B, and over 689 unique mutations are listed in an extensive mutation database of 1918 families who have been studied from around the world [6, 11]. Most of the unique mutations are point mutants causing missense, nonsense or splicing defects involving all 8 exons. For example, there are at present 425 different missense mutations that cause amino acid substitutions. These include the original case of Christmas disease, which is a G → C mutation at nucleotide 30,070 causing an amino acid change from Cys → Ser at residue 206 within exon 7 [12]. Many mutations have been observed more than once, even where there is no known kinship. Many of these repeat mutations – as in the case of haemophilia A patients, occur at CG doublets, suggesting they are independent mutation hotspots. However, other repeat mutations do not involve CG doublets, suggesting a founder effect. A good example is the mutation at nucleotide 31,311 where there are 41 examples.

A few patients have been described in whom the factor IX level rises significantly after puberty (the haemophilia B Leiden patients); this is associated with complete clinical recovery. These interesting regulatory mutations have been all localized to a short region of the factor IX promoter and 18 unique mutations are now known [6]. These mutations inhibit the binding of transcription factors, e.g., hepatic nuclear factor 4, which are required for the efficient initiation of transcription. An androgen response element (ARE) in the factor IX promoter, which interacts with the androgen receptor in the presence of testosterone, is responsible for the upregulation of factor IX mRNA at puberty [13]. Consistent with this hypothesis, patients with mutations in the ARE do not recover at puberty [13].

Treatment of haemophilia

Over a century ago, the first effective product available for treatment of bleeding episodes was fresh blood. The preparation of an antihaemophilic factor of bovine origin by Macfarlane in 1954 [14] was a major therapeutic advance, although serious allergic reactions were not infrequent. The use of cold-insoluble cryoprecipitate was introduced in 1965. The subsequent development of lyophilized concentrates of factors VIII and IX in the early 1970s transformed the life of haemophiliacs. The goals of treatment, as stated by the World Federation of Haemophilia, are "to minimise disability and prolong life, to facilitate general social and physical well-being and to help each patient achieve full potential whilst causing no harm" (www.wfh.org). Advances in therapy have certainly resulted in a dramatic increase in the longevity of haemophiliacs in developed countries. A study of 717 Dutch patients documented a calculated life expectancy for these patients of 66 years, compared with 74 year for normal males [15], and the authors of this study concluded that the mortality associated with even severe haemophilia was similar to that associated with cigarette smoking.

Treatment of bleeding episodes involves the intravenous injection of coagulation factor concentrates. The total dose and frequency of treatment is determined by the severity and site of bleeding. Most bleeds resolve with a single infusion, if the bleed is recognised early and treated promptly. There is an increasing move to prophylactic therapy, in which the patient gives himself injections at home of coagulation factors two or three times a week to prevent bleeds rather than just treating on demand when bleeds occur. Patients on prophylactic therapy experience few or even no spontaneous bleeds and thus progressive joint damage and arthritis can be avoided.

The pooling of plasma donations introduced the risk of transmission of viral infections, and many haemophiliacs were infected with HIV and/or hepatitis C in the 1980s. In the UK, 1229 haemophiliacs were infected with HIV, and about 300 were exposed to hepatitis C [16, 17]. The introduction in 1985 of physical methods of viral inactivation, such as heat-treatment or the addition of a solvent/detergent mixture, eliminated the risk of transmission of HIV or hepatitis C. However, other viruses are relatively resistant to these measures and cases of hepatitis A and parvovirus infection have been documented even with these more modern products. More recently, there has been concern about the possibility of transmission of prion disorders, although no cases have yet been described in haemophilia [18]. Recombinant products offer the greatest margin of safety for haemophiliac patients and have been recommended as the treatment of choice for all patients with haemophilia (see below).

Development of recombinant DNA methods

During the late 1970s there was a quiet revolution in molecular biology. Using restriction enzymes and DNA ligase, it became possible to clone copies of mRNA into either bacterial plasmids or into bacteriophages such as phage λ. Such clones could then be sequenced using Sanger's dideoxy chain termination method or Maxam & Gilbert's chemical degradation procedure. Once these mRNAs had been cloned, each cloned cDNA served as a probe in order to isolate from the entire genome its respective gene, which could then in turn be sequenced. Oligonucleotides were starting to be used as primers for the enzymatic synthesis of DNA. The presence of introns in most genes was discovered. Southern had described his blotting technique that was to become so widely used in this field until the development of PCR techniques much later in the 1980s.

Globin, immunoglobulin and ovalbumin mRNA – all mRNA species which could be purified in reasonable quantities from specialized cells or organs – could now be cloned in *E. coli* and sequenced in their entirety for the first time. Moreover, the new cloning methods suggested that mRNA and/or genes of medical interest, such as human insulin and human growth hormone mRNA, might be cloned and the protein expressed artificially in bacteria. This, it was thought, might be a cost-effective and safer alternative to isolating these proteins from natural sources for the treatment of diabetes and pituitary dwarfism.

1980 was a time of optimism and some of us thought that we might be able to clone rare, low-abundance mRNA, if only new methods could be developed. We decided to clone human factor IX mRNA. This would be an ambitious and difficult project because of its low abundance in liver. However, if the project were successful, the benefits for patients with haemophilia B would be considerable.

Recombinant factor IX

The factor IX gene was cloned in 1982 – some 2 years before the factor VIII gene – and this work will be described first. The expression of biologically active recombinant factor VIII, however, proved technically easier than factor IX. Moreover the priority was to produce recombinant factor VIII before recombinant factor IX because of the higher incidence of haemophilia A than B in the population. Consequently recombinant factor VIII was available for the treatment of patients in 1992, much sooner than recombinant factor IX, which was only produced much later in 1997.

Cloning the human factor IX gene in Oxford

It was the availability of the complete amino acid sequence of bovine factor IX [19] that initially led us to think that it might be possible to clone the factor IX gene. We thought that short synthetic oligonucleotides, with their sequence partly predicted from the genetic code and the amino acid sequence of the protein, would probably be the key to cloning rare mRNAs, like the factor IX mRNA. We had previously used oligonucleotides both for our earlier sequencing of regions within the α and β-globin, ovalbumin and immunoglobulin mRNAs [20], as well as for our later influenza cloning [21, 22]. Oligonucleotides were clearly powerful and highly specific reagents for nucleic acids. In particular, they could be used to hybridize to specific sequences in mRNA and prime the synthesis of cDNA by reverse transcriptase.

We first decided to enrich the bovine factor IX mRNA, which we had isolated from a calf liver, by two successive sucrose density-gradient centrifugation steps [23]. We then set up a rabbit reticulocyte *in vitro* translation assay to detect factor IX mRNA, locating it in the 20–22S fraction of the sucrose gradient. We estimated that it had been purified about 10-fold over the starting mRNA. Even so it was unlikely that the factor IX mRNA was >0.1% pure at this stage.

We then synthesized, as a mixture, eight 14-nucleotide (nt) long oligonucleotides (oligo N1 mixture), complementary to the bovine mRNA sequence, whose sequence was predicted from the amino acid sequence of residues 348–352 of bovine factor IX (Fig. 3). This was a particularly favourable region

```
First                   348             352
Amino acid              His-Met-Phe-Cys-Ala
sequence
                          U       U   U
mRNA                5'  CA  AUG UU  UG  GCN  3'
                          C       C   C

                          A       A   A
Oligonucleo-        3'  GT  TAC AA  AC  CG   5'
tides (N1)                G       G   G




Second                70                  75
Amino acid            Glu-Cys-Trp-Cys-Gln-Ala
sequence
                        A   U       U   A
mRNA              5'  GA  UG  UGG UG  CA  GCN  3'
                        G   C       C   G

                        T   A       A
Oligonucleo-      3'  CT  AC  ACC AC  GTT CG   5'
tides (N2A)             C   G       G

                        T   A       A
Oligonucleo-      3'  CT  AC  ACC AC  GTC CG   5'
tides (N2B)             C   G       G
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Figure 3. Two regions of amino acid sequence of bovine factor IX used to design mixed oligonucleotide sequence primers (N1) or probes (N2A & N2B) [22]. See text for further details.

of amino-acid sequence since the number of alternative possible nucleotide sequences predicted from the genetic code was limited to 8, because the sequence contains a methionine residue which has a unique codon. This oligo N1 mixture of primers was used to prime the synthesis of cDNA from factor IX-enriched bovine mRNA in order to generate a library of cDNA clones. Briefly, after synthesis of cDNA by reverse transcriptase and subsequent removal of mRNA by alkaline hydrolysis, "loop-back" synthesis of double-stranded DNA was catalysed by *E. coli* DNA polymerase I (Klenow subfragment) in the absence of added primer. Cloning of this double-stranded DNA was simplified by restriction digestion of DNA with MboI (GATC-recognition sequence) and ligation into the unique Bam HI site of the classic plasmid, pBR322. An "MboI" cDNA library of about 7000 recombinant clones was thus obtained in *E. coli*.

Bacterial colonies were then screened on Whatman 541 paper [24] using a second mixture (oligo N2A plus oligo N2B) of 16 ^{32}P-labelled 17-nt long oligonucleotides based on the predicted sequence of bovine factor IX mRNA of another favourable region of amino acid sequence between residues 70–75 (Fig. 3). We obtained a single clone, labelled BIX-1, which was sequenced by the Maxam and Gilbert method. This clone encoded bovine factor IX mRNA from amino acids 52 to 139 and predicted an amino acid sequence that was in complete agreement with the literature, except for a single discrepancy changing the amino acid at position 57. This discrepancy was, in fact, confirmed as a correction to the published bovine factor IX sequence, since it was also present in an independent clone in a "dC/dG-tailed" cDNA library of about 10,000 *E. coli* colonies.

Thus the choice of oligonucleotides and our ability to synthesize them in-house were two important factors in our initial success in isolating factor IX cDNA clones. Acting in the hope that the human and bovine factor IX genes would be sufficiently conserved in nucleotide sequence to cross-hybridize, the bovine factor IX probe was then used to probe a Charon 4A bacteriophage λ library of human genomic clones [22]. A positively hybridizing clone, λHIX1b, was isolated and mapped by restriction enzyme cleavage and Southern blotting. A restriction fragment corresponding to the probe was thus identified on Southern blots and sequenced. There was 85% nucleotide sequence conservation between bovine and human factor IX gene in the region initially sequenced. We had thus isolated a clone containing part of the human factor IX gene.

Further cloning and expression of factor IX in mammalian cells

Further cloning Two additional reports from other groups [25, 26] extended our initial work by describing the complete coding sequence of human factor IX, which was found to be preceded by a leader sequence with a potential signal peptide and propeptide sequence. There was uncertainty, however, as to whether the entire coding sequence had been cloned, since the 5' non-coding of the factor IX mRNA was not characterized. There were, also, in one report [26] some 6 differences in the coding nucleotide sequence attributed, incorrectly as it turned out later except for one nucleotide [27], to the presence of polymorphisms. Interestingly, although the details of the cloning procedures differed in these reports from our own work, all of the studies had relied on oligonucleotides to prepare or isolate clones from cDNA libraries.

We concentrated on characterizing the complete factor IX mRNA sequence by cDNA cloning, and independently sequencing all exons in order to define splice points and to check for potential cDNA cloning errors, which are known to occur during reverse transcription and cloning. We defined the mRNA start site precisely and sequenced the entire 3' non-coding region of the mRNA [27]. The factor IX gene turned out to be about 34 kb long with 8 exons. The factor IX mRNA was about 2800 nucleotides long and had a relatively short 5' non-coding region, but a reasonably long 3' non-coding sequence of nearly 1400 nucleotides containing the usual AAUAAA poly(A) signal. Finally, the complete human factor IX gene sequence of 33.5 kb, including all 7 introns, was established by Davie's group [28].

Expression of biologically active factor IX in mammalian cells If cloning the factor IX cDNA and gene sequence were to be a "useful" contribution to medicine, then expression of recombinant factor IX protein would have to be achieved. A major uncertainty was whether *biologically active* factor IX was synthesized, because after synthesis, factor IX has to be post-translationally modified and correctly processed. In particular, after γ-carboxylation of 12 glutamyl residues near the N-terminus of factor IX by a vitamin K-dependent carboxylase, the 18 amino acid residues of the propeptide sequence [29] must be cleaved by a furin protease. Both these processes are essential for factor IX activity. It was conceivable that these essential post-translational events would only occur in liver, the site of factor IX synthesis in the body, and would not occur in hepatic or other cell types derived from other tissues in tissue culture.

We were fortunate that the aim of producing biologically active factor IX in mammalian cells in tissue culture was feasible but it was only achieved after a considerable effort by Don Anson and Ian Jones in the Brownlee laboratory. We published, in 1985, that "we were able to isolate small amounts of biologically active (recombinant) human factor IX" [30]. In particular, factor IX was expressed in rat hepatoma cells that had been stably transfected with a factor IX expression plasmid. Much higher yields of >90% biologically active factor IX were obtained in canine kidney cells (MDCK cells) using a slightly different expression plasmid [31]. Our results were particularly convincing because we purified the recombinant factor IX by immuno-affinity chromatography.

Subsequently, higher yields of recombinant factor IX were achieved by other groups using different expression vectors in different mammalian cells, i.e., baby hamster kidney (BHK), human hepatoma (HepG2) and chinese hamster ovary (CHO) cells [32–34]. But in every case the biological activity of factor IX was less than in our reports. Indeed, in the initial work by Kaufman's group in CHO cells (in which the factor IX gene was amplified in the genome) factor IX was secreted to give as much as 100 mg/ml of factor IX in the medium, yet only 2% was biologically active [34]. This low biological activity in CHO cells was subsequently solved by the introduction of a furin-type protease (see below), suggesting that factor IX propeptide processing must have been limiting.

Development of recombinant factor IX for clinical use

The observation that some, albeit low, biological activity was present in the factor IX expressed in CHO cells was important [34], since this cell line has properties that are particularly suited for large-scale culture needed for the industrial production of recombinant proteins by the biotechnology industry. Therefore, development work was undertaken by Genetics Institute, Boston. This presented a significant challenge since it was essential that propeptide processing and γ-carboxylation, known to be essential for factor IX activity, had to occur. Neither of these processes had been previously required in CHO cells in the production of recombinant proteins for the biotechnology industry.

Initially it was suspected that γ-carboxylation was limiting in the factor IX-expressing CHO cells, thus accounting for the presence of the inactive, incompletely γ-carboxylated factor IX. However, when the cloned γ-carboxylase became available, transient transfection of expression constructs of the γ-carboxylase into factor IX-secreting CHO cells failed to improve factor IX biological activity [35]. By contrast, propeptide processing of factor IX was significantly improved by the co-expression of a furin-like enzyme, specifically a soluble form of PACE (paired basic amino acid cleaving enzyme) introduced stably into the CHO cell line [36]. Finally, Genetics Institute produced factor IX in a form that was fully processed by screening factor IX-expressing CHO clones to identify those with the highest factor IX-processing capacity, by optimizing the concentration of vitamin K added to the defined serum-free culture medium (which lacked any added protein) and by defining the precise conditions of growth of the factor IX-secreting CHO clone in large-scale (2500 l) bioreactors.

A down-stream large-scale purification protocol was developed that did not require the use of monoclonal antibodies for affinity purification, to avoid any risk of introducing viral contaminants. The purified recombinant factor IX showed a slightly reduced γ-carboxylation level of about 11 γ-carboxyglutamyl (Gla) residues per molecule compared to 12 Gla residues in blood-derived factor IX (Tab. 1 and ref. [37]). A new result obtained from the analysis of this recombinant factor IX is that the post-translational modification of Gla residues 36 and 40 are not apparently needed for activity [38]. Other differences between recombinant and human blood-derived factor IX were appar-

Table 1. Comparison of post-translational modifications of plasma-derived and recombinant factor IX

Modification	Plasma-derived	Recombinant
1. γ-carboxyglutamic acid (Gla)*		
12 of 12 Gla residues	100%	60%
11 of 12 Gla residues	0%	35%
10 of 12 Gla residues	0%	5%
2. β-hydroxyaspartic acid 64	37%	46%
3. Carbohydrates		
N-linked glycans		
Asn 157 Asn 167	High heterogeneity fully sialylated	Low heterogeneity <fully sialylated
O-linked glycans		
Ser 53	$(Xyl)_{1-2}$-Glc	$(Xyl)_2$-Glc
Ser 61	NeuAcGalGlcNAcFuc	NeuAcGalGlcNAcFuc
Thr 159, 169 & 172	Classical, partially filled	Classical, partially filled
4. Tyr 155 sulphation	>90%	<15%
5. Ser 158 phosphorylation	>90%	<1%

Footnotes: Adapted from ref. [37] with permission. *The 10 completely modified Gla residues occur at amino acid residues 7, 8, 15, 17, 20, 21, 26, 27, 30 and 33. Gla residues at positions 36 and 40 are incompletely modified, as indicated, in recombinant factor IX.

ent in the extent of N- and O-linked carbohydrate side chains, and in sulphation and phosphorylation levels of particular residues (Tab. 1). The extent of β-hydroxyaspartate modification at serine 64, however, was similar. Fortunately, none of the differences in post-translational modification seemed to affect the biological activity of factor IX, although the differences in sulphation and phosphorylation, it was suggested, may explain the slightly lower recovery (on average about 20% lower) of recombinant, as compared to blood-derived, factor IX seen in patients [37].

In summary, recombinant factor IX is already really a "second-generation" recombinant product because it is prepared from cultured cells and purified under conditions where there is no contact with human or bovine proteins [37]. Thus the product should be free of known blood-borne viral diseases, such as HIV, hepatitis A, B or C and parvoviruses. There should also be no risk from diseases caused by prions, such as Creutzfeldt-Jakob disease (CJD) or the variant CJD, caused by the prion of bovine spongiform encephalitis in cattle. Recombinant factor IX (BeneflX) was approved for sale in the USA and Canada in 1997 and in Europe in 1998.

Recombinant factor VIII

When factor VIII was first cloned and expressed in cultured cells by two competing companies in 1984, this achievement was hailed as one of the most exciting advances to be reported by the biotechnology industry [39]. However, in a Nature editorial in the same week that the factor VIII cloning was announced, we were poignantly reminded that the first deaths had occurred in Australia and in the UK from HIV-contaminated blood. Clearly blood-derived clotting factors were potentially dangerous and would eventually be superseded by recombinant proteins. Not for the first time was the fear of disease driving scientists and biotechnology companies to find improved and safer medicines.

Cloning and expression of factor VIII

The factor VIII gene and its cDNA were cloned by the use of oligonucleotide probes based on the amino acid sequence of peptides isolated from either human or porcine factor VIII, essentially by similar methods to those employed previously to clone factor IX. Due to its low concentrations in plasma, its high molecular weight and extreme sensitivity to proteolytic processing, it was a very difficult protein to purify. The breakthrough came when amino acid sequence information on peptide fragments of human or porcine factor VIII became available [40, 41].

Surprisingly, a unique 36-nt long oligonucleotide, rather than a mixture of oligonucleotides, was successfully used as a probe in one report [40], and in the other [41] two sets of mixed oligonucleotides, either 45-nt long or 15-nt long, were used. Because of uncertainty as to whether factor VIII was synthesized in the liver, both groups initially cloned the factor VIII gene by screening genomic libraries of clones in bacteriophage λ with these probes. The gene turned out to be 180 kb long with 26 exons and at that time was the longest known gene. Human cDNA clones were then subsequently isolated, using genomic probes, by screening appropriate human cDNA libraries. Full-length cDNAs were then used for expression studies by introducing expression plasmids with heterologous viral promoters into BHK [40] or monkey kidney (COS-1) cell lines [41]. Human factor VIII was secreted into the medium of these transfected cells and shown to be active in a clotting assay. Cloning and characterization of the factor VIII gene and its cDNA had been a long task, but its expression was initially surprisingly straighrforward.

Development of recombinant factor VIII for clinical use

The subsequent production and purification of recombinant factor VIII from cultured mammalian cells by the biotechnology industry were quite rapid and commensurate with the urgency in producing recombinant factor VIII, which was free of viral contamination for the treatment of haemophilia A patients.

Initially, however, there was some difficulty in the approach developed by Kaufman's group at Genetics Institute in obtaining high yields of factor VIII secreted by CHO cells. Factor VIII cDNA had been introduced into CHO cells by linking it to the selectable, amplifiable marker, dihydrofolate reductase (DHFR). Protein expression levels were, however, much lower than had been observed with other cDNAs, e.g., factor IX, in CHO cells. It emerged that the reason for this was that during intracellular processing of factor VIII in the ER, a significant proportion of the factor VIII became bound to the chaperone, immunoglubulin-binding protein (BiP), through a primary binding site of its A1-domain. Interactions also occurred between factor VIII and 2 other protein chaperones, calnexin and calreticulin, primarily mediated by the B-domain of factor VIII. It was suggested that factor VIII has unique requirements, not shared by the related coagulation factor V, for carbohydrate processing and molecular chaperone interactions that may limit its secretion [42]. In practice the problem of the low yield of secreted factor VIII was initially overcome, at least in part, by the addition of von Willebrand factor (vWF) (the cofactor with which factor VIII is normally associated as a high-molecular-weight complex in plasma) to the tissue culture medium. Thus factor VIII secreted in the absence of vWF appeared to be rapidly degraded. In its presence, however, factor VIII associated with vWF to form high-molecular-weight multimers. This stabilized the factor VIII, protecting it from proteolytic degradation and improving factor VIII yields significantly [43].

Finally, in order to develop a large-scale process for production of factor VIII, the vWF cDNA was introduced into the factor VIII-expressing CHO cells by linking it to a second amplifiable marker, the adenosine deaminase gene. Co-expression of factor VIII and vWF was thereby achieved even in serum-free medium (Fig. 4), improving the factor VIII yields by 1–2 orders of magnitude [44, 45]. Even so, the yield of factor VIII was still significantly lower

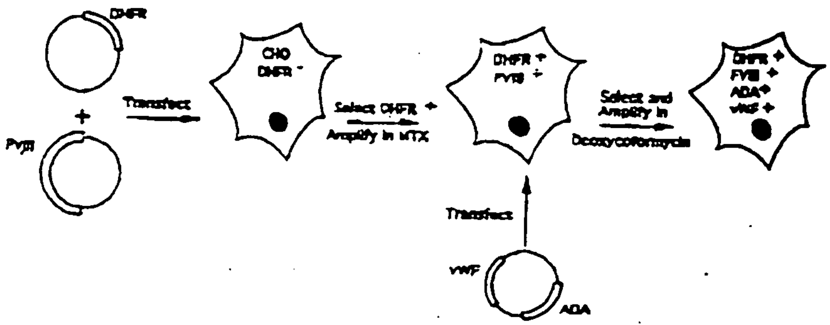

Figure 4. Derivation of factor VIII and von Willebrand factor coexpressing CHO cells used for the manufacture of recombinant factor IX. Adapted from Kaufman [45] with permission. Plasmids encoding DHFR and factor VIII were transfected into DHFR-deficient CHO cells and transformants selected by growth in nucleoside-free medium. Cells were then selected in increasing concentrations of methotrexate to obtain a cell line that had amplified the DHFR and factor VIII genes. These cells were subsequently transfected with a vWF expression plasmid containing an ADA (adenosine deaminase) gene and selected for growth in the presence of cytotoxic concentrations of adenosine with increasing concentrations of 2'-deoxycoformycin. The final cell expressed DHFR, ADA, vWF and factor VIII.

than that of the co-expressed vWF. This suggested that the intracellular regulation of factor VIII mRNA levels and/or regulation of the intracellular folding and processing of factor VIII were still limiting the yields of factor VIII secreted into the culture medium [44]. The factor VIII-expressing CHO cell line is cultured in a defined, serum-free medium in 2500 l bioreactors for the large-scale manufacture of recombinant factor VIII. However, it should be noted that the culture medium is reported to contain (unspecified) added bovine proteins from natural sources, which were, however, tested to minimize the risk of introducing viral contamination [46].

By contrast, a different cell line was used for factor VIII expression in the approach adopted by Genentech/Bayer. A clone of BHK cells was isolated after introducing the factor VIII cDNA, linked to DHFR, into chromosomal DNA followed by amplification of the factor VIII copy number by selection with methotrexate. The BHK clone used for the commercial production of factor VIII contains about 150 copies of the factor VIII cDNA per cell [47]. For the subsequent manufacture of factor VIII in deep-tank stirred suspension fermenters, a proprietary (unspecified), serum-free medium was used [48]. This medium presumably contained some added protein, since there was no requirement to introduce the vWF cDNA to improve the stability of the factor VIII secreted in this BHK cell line, unlike the situation in CHO cells (see above).

Both recombinant factor VIII products (Genetics Institute or Genentech), after purification by immuno-affinity chromatography with monoclonal antibodies, ion-exchange chromatography and other methods, were reported to be essentially indistinguishable from plasma-derived factor VIII [45, 48]. The full-length recombinant factor VIII produced by both companies may be regarded as a "first-generation" product, since the CHO cells used to produce factor VIII were grown in a tissue culture medium that contained protein additives. Moreover, the resultant purified factor VIII was stabilized by the addition of human albumin. Thus there remains a small theoretical risk, since natural products from humans or cattle were used in their manufacture, that heat-resistant viruses, such as B19 parvovirus (causing fifth disease) or, more seriously, prions, might still be present. Approval was granted in 1992 for the sale, in the USA, of Recombinate (manufactured by Genetics Institute/Baxter) and in 1993 for the sale of Kogenate (manufactured by Genentech/Bayer, originally Miles/Cutter).

B-domainless recombinant factor VIII

A second generation, B-domainless factor VIII, recombinant factor VIII SQ ("Refacto": Pharmacia/Upjohn/Genetics Institute), is now (1999) also approved [49]. Early work, both by recombinant DNA and classic protein chemistry methods, had shown that factor VIII with its B domain deleted was active in clotting assays [50–52]. Factor VIII SQ was developed from one particular construct, which retained the factor VIII processing protease cleavage sites at amino acids 740 and 1649 with only 14 residues of the B domain still present. This was almost fully processed when expressed in CHO cells, consistent with its retaining a furin cleavage site preceding amino acid 1649 [53]. An advantage of factor VIII SQ, in comparison to the first-generation full-length factor VIII products, is that it is formulated without the addition of human albumin. However, human albumin is still added to the tissue culture medium in which the CHO-expression construct is grown [49]. Thus, these second-generation products have a reduced theoretical risk of transmission of human viruses [49].

Clinical experience with recombinant factors

Recombinant products offer the greatest margin of safety for haemophilic patients and have been recommended for the treatment of all patients. However, they are significantly more expensive and many patients continue to receive plasma-derived products. In addition to offering an increased margin of safety with regard to viral contamination, recombinant products also offer a solution to the problem of the burgeoning demand for coagulation factors which continues to rise steeply. Thus 62 million units of factor VIII were used in the UK in 1981, but by 1998 consumption had increased to 200 million units. This largely reflects the changing pattern of treatment, with patients now being encouraged to treat themselves at home on a prophylactic basis to prevent joint bleeds. The plasma half-life and recovery of the various recombinant factor VIII products are identical to those observed with conventional plasma-derived products and are typically around 14–16 hours. In contrast to many plasma-derived products, none of the recombinant products contain vWF, and thus they are of no use in the treatment of this different congenital haemorrhagic disorder. The plasma half-lives of recombinant factor IX are approximately 18 hours, which are also the same as those observed with plasma-derived factor IX products [37].

Recombinant factor VIIa

Recombinant factor VIIa (Novoseven, Novo Nordisk) has proved very useful in the clinical management of patients with either haemophilia A or B and inhibitory antibodies, as well as those with acquired haemophilia. It by-passes the requirement for either factor VIIIa or IXa because it activates factor X (see Fig. 1). Control of bleeding episodes in patients with inhibitory antibodies is a major clinical challenge. Most of the clinical experience relating to use of recombinant factor VIIa has been gained outside the setting of formal clinical trials, and this has hindered licensing in the United States [54]. The product is extremely expensive and this is likely to hinder widespread use when other products may be just as effective. It is valuable in patients who have high titres of inhibitory antibodies. It is of particular value, however, in the few patients with haemophilia B and inhibitory antibodies, as administration of either plasma-derived or recombinant factor IX in these cases can result in serious anaphylactic reactions [55]. Factor VIIa has a plasma half-life of only approximately two hours, so that frequent bolus injections are required to control bleeding; it is not licensed for continuous intravenous administration. No specific adverse effects have been identified. The excellent safety profile, including lack of allergic reactions, has encouraged some clinicians to employ this product for first-line treatment of bleeds at home [56]. In the past, physicians were often reluctant to undertake elective surgery in haemophiliacs with inhibitors, but such procedures can now be carried out with confidence with factor VIIa [57].

Safety issues

Concerns about recombinant coagulation factor concentrates have focused on three main issues: viral safety, incidence of inhibitor development and problems with laboratory assays. It is somewhat ironic that several currently licensed preparations of recombinant factor VIII actually contain a considerable quantity of human, plasma-derived albumin that is added to the product as a stabilizer. Furthermore, most recombinant products are not subjected to specific virucidal treatment, such as heat treatment, during manufacture. Alternative stabilizers have been developed and clinical trials with these second-generation recombinant factor VIII products are already underway. Recombinant factor IX does not require the addition of albumin as a stabilizer. Bovine proteins are incorporated in growth media used in the manufacture

Table 2. Recombinant factor concentrates

Product and manufacturer	Viral inactivation	Human albumin	Bovine protein
Recombinate (Baxter)	No	Yes	Yes
Kogenate[1] (Bayer)	Yes	No[2]	No
Refacto (Wyeth)	Yes	No[2]	No
BeneFIX (Baxter)	Yes	No	No
Novo Seven (Novo)	No	No	Yes

Footnotes: [1] Identical to Helixate (Aventis); [2] although cells are grown in human albumin

of some products (see above). The current status of the various recombinant products is summarized in Table 2.

Concern has also been expressed that the use of highly purified factors might result in a greater incidence of inhibitory antibodies. Unfortunately, it is not possible to give a definitive answer to this important issue, as no prospective, double-blinded clinical studies have been conducted in which the incidence of inhibitor development has been compared in patients receiving recombinant products and those receiving conventional plasma-derived products. Approximately 5% of patients with haemophilia A in the UK are known to have developed inhibitory antibodies (or about 15% if severe patients with a baseline of <2% factor VIII are considered). The first two clinical trials of two different recombinant products in previously untreated patients (PUPs) reported an incidence of inhibitor development of around 20% [58, 59]. Inhibitors appeared after a mean of 9 exposure days in both clinical trials. A more recent study from France with a longer follow-up period reported an inhibitor incidence of 28% in patients receiving recombinant factor VIII, in contrast to an incidence of 9% in a historical control group which received only plasma-derived products [60]. Similar results have been observed in other groups. Most of the cases involved low titre and/or transient inhibitors that disappeared as treatment was continued. It is quite probable that many cases of transient or low-titre inhibitors amongst patients receiving conventional concentrates were simply not identified in the past, and most haematologists do not believe that the incidence of significant inhibitors in patients receiving recombinant products is truly elevated. The median number of exposure days until detection of the inhibitor was 9.5 days (range 5–14 days).

By contrast with recombinant factor VIII, there is no evidence of an increased risk of inhibitor development with the use of recombinant factor IX. In clinical trials, only one of 31 PUPs developed an inhibitor and only one of 56 previously treated patients (PTPs) developed a transient inhibitor. These findings are comparable with the incidence of inhibitors of approximately 1% of historical controls with haemophilia B. The formation of specific antibodies against recombinant factor VIIa has not been identified in subjects with either congenital or acquired haemophilia.

The ability to assay the activity of factor VIII both in actual coagulation factor concentrates and in the plasma of haemophiliac patients after infusion of products is vital for clinical care. Various methods have been developed for the assay of factor VIII, including the one-stage, two-stage and automated chromogenic methods. In general, clinical laboratories tend to prefer the one-stage method, which is both faster and easier to perform, for assay of factor VIII in both concentrates and patient plasma. By contrast, the potency of concentrates is now assigned by the chromogenic method, which is actually based on the two-stage method. Traditionally, human plasma is used as the standard for assays of plasma factor VIII. When plasma levels of factor VIII are assayed after infusion of recombinant factor VIII, recovery is significantly greater when using the chromogenic assay compared to the conventional one-stage assay. Pharmacokinetic evaluation also shows a 25% greater AUC (Area under curve (concentration × time) with the chromogenic method, but half-lives are identical on calculations using either assay method. It has recently been shown that these differences may be resolved by using recombinant concentrate diluted in haemophilic plasma as the laboratory standard. The recovery of recombinant factor IX in patients is slightly lower (on average by about 20%) than that observed with standard, plasma-derived products (see above). This means that a higher dose has to be given to patients to achieve a defined target plasma-level. Marked differences in laboratory assay results have been observed with "Refacto" (B-domainless factor VIII). The potency of this product should be assayed using the chromogenic substrate method, since conventional one-stage assays usually underestimate the real potency.

Summary and prospects

Recombinant factors VIII, IX and VIIa are now available for treatment of haemophilia A & B patients in countries where this newer form of "protein therapy" is affordable. They are safer than plasma-derived factors because they carry no, or minimal, risk of contamination by viruses. Unfortunately, at present, they are more expensive. In the future, gene therapy for haemophiliacs may become available. This subject has certainly been widely discussed as a potential cure for patients and there is considerable current interest in this topic [61]. Good progress has been made with adenoviral and adeno-associated viral vectors and clinical trials are in progress with adeno-associated viral vectors. Thus gene therapy is some way off and may not become available for haemophiliacs for some time.

Acknowledgments
G.G.B. thanks Dr. P. Handford, Dr. E.J. Boswell, Dr. R.J. Kaufman, Dr. J. Edwards, Dr. M. Mikaelsson and Prof. E.G.D. Tuddenham for critical comments.

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Factor Concentrates

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Factor VIII Concentrates

Description

Factor VIII concentrates are a commercially prepared, lyophilized powder purified from human plasma to treat patients with hemophilia A or von Willebrand's disease. Alternatively, recombinant (synthetic) protein is purified from genetically engineered non-human cells grown in tissue culture. The quantity of factor VIII coagulant activity is stated on the bottle. One factor VIII concentrate unit equals the clotting activity in 1 ml of fresh plasma. Factor VIII concentrate is cell free and is administered without regard to patient or donor ABO or Rh type. It is heat treated and/or solvent detergent treated to reduce the risk of virus transmission. Current processes appear to have eliminated the risk of HIV, HBV and HCV transmission. Concentrates differ in the purification procedures. Highly purified factor VIII, e.g. preparations purified over a monoclonal antibody column or current recombinant factor VIII concentrates, are stabilized by adding 98% of pasteurized human albumin. Porcine factor VIII concentrate is available for patients with high titer anti-human factor VIII allo or autoantibody inhibitors. Factor VIII concentrates are stored refrigerated at 2-8°C for up to two years from the date of manufacture (expiration date will be indicated on each vial). Some preparations may be kept at room temperature for extended periods. Once reconstituted, it should not be refrigerated. Factor VIII concentrate should be infused within 4 hours of preparation to reduce the risk of bacterial growth. Vials are usually shipped to a hospital pharmacy, blood service or nursing unit and mixed there prior to use. Many patients or families receive them directly for home care.

Indication

Factor VIII concentrate is indicated for the treatment of bleeding or imminent invasive procedures in patients with hemophilia A (congenital factor VIII deficiency) and for patients with low titer factor VIII inhibitors. Regular prophylactic doses are sometimes used as well as daily doses in some hemophilic inhibitor patients to try to induce immune tolerance. Patients with von Willebrand's disease respond to one specific, pasteurized intermediate-purity concentrate in which von Willebrand factor activity is relatively preserved.

Therapeutic Effect

Dosage is dependent on the nature of the injury, the degree of factor deficiency, the weight of the patient and the presence and level or absence of factor VIII inhibitors. The half life of circulating factor VIII is 8 to 12 hours, therefore transfusions may need to be repeated every 12 to 24 hours to maintain hemostatic levels. Following surgery, it is necessary to maintain hemostatic levels for up to two weeks to prevent delayed bleeding and promote wound healing in the hemophilic patient. The Puget Sound Blood Center's Hemophilia Program is available for consultation at 206-292-6507 (or 1-800-552-0640) or 206-292-6525 (and page) on evenings or weekends.

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Emory Health Sciences Press Release

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EMORY'S COMPREHENSIVE HEMOPHILIA PROGRAM IS A LEADER IN CLINICAL RESEARCH AND ADVANCED TREATMENTS FOR ADULTS AND CHILDREN

The comprehensive hemophilia program at Emory University has become a national leader in research and treatment programs that are helping improve the lives of the 15,000 hemophiliacs living in the U.S. Although these patients now can enjoy a normal life span, they must rely on infusions of blood products to treat frequent bleeding episodes, then cope with resulting complications, including inhibitors that render the products ineffective and the threat of infections and joint diseases caused by internal bleeding. In the past, the cure for hemophilia has sometimes been worse than the disease.

Emory hematologists participate in a number of clinical trials to improve treatments for hemophilia patients, including a recently concluded international study sponsored by Bayer to test a new formulation of blood clotting products. The new formula, called Kogenate FS, is almost completely free of any human or animal components and should make hemophiliacs feel safer than ever about using genetically engineered clotting factors. Kogenate FS has recently been approved by the Food and Drug Administration.

Individuals with hemophilia are missing the gene that makes Factor VIII, a critical part of the blood clotting machinery. Factor VIII is a component of fibrin, which is the cement the body uses to seal a wound after platelets first plug it up, much like the Dutch boy putting his finger in the dike while awaiting help.

In the past, Factor VIII products to treat hemophilia patients were made by concentrating clotting factor gathered from the plasma of a large group of donors. In the mid 1980s, when scientists discovered that these blood products could transmit diseases like HIV and hepatitis C, they began heating Factor VIII products to kill these viruses.

In the early 1990s, scientists carried safety one step further with genetically engineered recombinant Factor VIII products made by inserting the factor VIII gene into a cell line and producing mass quantities of concentrated human factor VIII. Although these products contained no human or animal products, they were stabilized with a small amount of albumin, a human blood component. Kogenate FS -- the newest FDA-approved product -- uses small amounts of albumin in the initial "fermenting" solution, but in the final stage, albumin is removed, leaving the product almost completely free of any human or animal components. The Factor VIII is then stabilized with sucrose. The product is said to be sucrose-formulated and albumin-free in final formulation.

"Our research found that the new product works just as well as the current products and appears to offer a greater safety margin against infectious agents," says Thomas Abshire, M.D., medical director of Emory's hemophilia program and one of the principal investigators for the study.

Emory has just completed another randomized study, in collaboration with Schering Plough, Inc. and the American Red Cross, in which hemophilia patients with hepatitis C were treated either with a combination of interferon and Ribavirin, or with interferon alone, which is the known treatment. Preliminary results presented at the World Federation of Hemophilia in July determined that the combination therapy is better than interferon alone.

Emory also is a world leader in treating joint disease in hemophiliacs -- a common problem caused by bleeding into joints, which causes irritation in the lining of the joint cavity and creates a cycle of bleeding and inflammation. Surgeons and hematologists have collaborated on a study of arthroscopic synovectomy, in which a small endoscope is inserted into the ankle, elbow, or knee to clean out the thickened lining. When surgery is not an option, physicians use an alternative technique called radionuclide synovectomy to inject a radioisotope into the joint that eliminates the abnormal lining. Drs. Michael Busch and Amy Dunn coordinate this program.

Emory's comprehensive adult and pediatric hemophilia program includes hematologists, infectious disease specialists, hepatologists, orthopaedic surgeons, physical therapists and specialty nurses. The program receives some federal funding through the Maternal and Child Health Bureau (MCHB) and the Centers for Disease Control and Prevention (CDC). Two adult and two pediatric hematologists treat 350 patients, including 140 children. The staff also works closely with a program at Children's Healthcare of Atlanta at Scottish Rite, which treats 150 additional children. Dr. Abshire also is Medical Director for MCHB Region IV South hemophilia programs that include Alabama, Mississippi, Georgia and Florida.

Although the number of hemophilia patients is small relative to many other diseases, it commands a great deal of attention because it is so expensive to treat. Clotting factor for a mild joint bleed in a typical 7-year-old child, even at reduced rates, averages $600 per infusion, with some patients needing several infusions per week. "There is a motivation to produce a better product and one you can use less of, which may come with the eventual development of gene therapy for Factor VIII," says Dr. Abshire.

Other current clinical trials at Emory aimed at treating bleeding disorders and their complications include: (1) a study of children who experience clotting problems from permanent IVs; (2) a CDC-sponsored study designed to identify and treat women with undiagnosed bleeding disorders who are experiencing abnormal bleeding with menstrual periods (hematologist Sidney Stein, M.D., leads the Emory component of this multi-site study); (3) multiple clinical trials designed to evaluate the safety and efficacy of new products used to treat bleeding episodes; (4) multiple AIDS Clinical Trials Group (ACTG) studies for our patients that contracted HIV infection from blood products prior to the development of safer products.

The Emory hemophilia treatment center also participates in studies designed to identify the safest and most cost effective methods for preventing the complications of congenital bleeding disorders, including the CDC-sponsored Universal Data Collection System and the National Hemophilia Foundation's National Prevention and Awareness Campaign.

Exciting research advances are on the horizon to deal with the problems faced by the 20 percent of hemophilia patients who have antibodies that inhibit the effectiveness of substitute Factor VIII products. Emory hematologist J.S. "Pete" Lollar, M.D., is conducting groundbreaking research in the laboratory that includes gene therapy and an improved Factor VIII molecule constructed from a combination of human and pig Factor VIII genes.

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Last Updated: October 13, 2000

NATIONAL HEMOPHILIA FOUNDATION
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News

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January 10, 2001

Refacto (rFVIII) to Become Available

Wyeth/Genetics Institute has announced that their second-generation recombinant factor VIII product, ReFacto will be available in the United States beginning on January 29, 2001. ReFacto is the first recombinant factor VIII product formulated without human serum albumin in its final formulation.

ReFacto is indicated for the control and prevention of hemorrhagic episodes and for surgical prophylaxis in patients with hemophilia A. ReFacto is also indicated for short-term routine prophylaxis to reduce the frequency of spontaneous bleeding episodes.

Wyeth/Genetics Institute claims that dosing for ReFacto will be exactly the same as the other second-generation recombinant factor VIII products currently on the market. Further, Wyeth/Genetics Institute has announced that 2000 I.U. sizes for both ReFacto and their recombinant factor IX, BeneFix, will be available.

Due to limited supply of ReFacto however, Wyeth/Genetics Institute will be placing limits on individual customer size orders. Wyeth/Genetics Institute has devised an allocation method whereas the 18 largest homecare companies and the hemophilia treatment centers will be allocated product based on the percentage of hemophilia A patients they see. For example, if ABC Homecare has a hemophilia A base of 10% of the overall hemophilia A population, they would be allocated 10% of Wyeth/Genetic Institute's inventory of ReFacto.

Increased production for ReFacto is expected sometime next year as Wyeth/Genetics Institute is working towards completion of a refurbished manufacturing plant in St. Louis, Missouri. Currently, product is being imported from a manufacturing facility in Stockholm, Sweden. The St. Louis facility is forecast for completion in early 2002.

The list price for ReFacto will be $1.09. However, Wyeth/Genetics Institute is going to offer a bulk contract price of $0.84. The list price for other second-generation recombinant factor VIII products are similar (Kogenate FS - $1.13; Helixate FS - $1.11).

The Average Wholesale Price (AWP) of ReFacto is expected to be the similar to other second-generation recombinant factor VIII's as well. Wyeth/Genetics Institute expects the

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AWP of ReFacto to be the list price ($1.09) multiplied by 25%. It this is the case, ReFacto's AWP would be $1.36 whereas Kogenate FS would be $1.41 and Helixate FS $1.38.

Wyeth/Genetics Institute is also working on a third-generation recombinant factor VIII named ReFacto AF (Albumin Free). The St. Louis manufacturing facility has been selected to product ReFacto AF, once the FDA approves that product. Wyeth/Genetics Institute has yet to decide if they will phase out ReFacto once ReFacto AF has been approved. In similar situations, Bayer chose to phase out their first generation recombinant product, Kogenate, once their second-generation product, Kogenate FS was licensed. However, Baxter Hyland Immuno has decided to keep their first generation recombinant product Recombinate on the market, even after their third-generation recombinant product is licensed.

Wyeth/Genetics Institute currently manufactures and distributes ReFacto in Europe. Moreover, they are currently the only manufacturers of a recombinant factor IX product, BeneFix.

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